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Molecular Analysis of Kinetochore-Microtubule Attachment in Budding Yeast

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Summary

The complex series of movements that mediates chromosome segregation during mitosis is dependent on the attachment of microtubules to kinetochores, DNA-protein complexes that assemble on centromeric DNA. We describe the use of live-cell imaging and chromatin immunoprecipitation in *S. cerevisiae* to identify ten kinetochore subunits, among which are yeast homologs of microtubule binding proteins in animal cells. By analyzing conditional mutations in several of these proteins, we show that they are required for the imposition of tension on paired sister kinetochores and for correct chromosome movement. The proteins include both molecular motors and microtubule associated proteins (MAPs), implying that motors and MAPs function together in binding chromosomes to spindle microtubules.

Introduction

The segregation of replicated sister chromatids into two equal sets at mitosis involves a complex series of movements mediated by kinetochores, DNA-protein complexes that assemble on centromeric DNA. Following microtubule attachment early in mitosis, paired sister chromatids exhibit directional instability and undergo oscillatory movements back and forth along spindle microtubules (Skibbens et al., 1993). Sister separation is delayed by a mitotic checkpoint comprising *MAD* and *BUB* genes that is silenced only when all pairs of chromatids have achieved bipolar attachment. Sister cohesion is then dissolved and chomatids begin their anaphase movement toward the spindle poles.

As structures that link centromeres to spindle fibers, kinetochores have both DNA and microtubule binding activities. The unusual compactness of *S. cerevisiae* centromeres (approximately 175 bp) has facilitated biochemical and genetic analysis of kinetochore-associated DNA binding proteins. These include CBF3 and the specialized H3 histone Cse4p (Stoler et al., 1995). The assembly of kinetochores in *S. cerevisiae* appears to begin with the binding of CBF3, a four-protein complex, to the essential CDEIII region of centromeric DNA. Cells carrying temperature sensitive mutations in CBF3 subunits (Ndc10p, Cep3p, Ctf13p, or Skp1p) experience greatly elevated chromosome loss under semipermissive conditions (Hyman and Sorger, 1995). Several additional proteins have been identified that bind to yeast centromeres in a CBF3-dependent fashion (Hyland et al., 1999; Meluh et al., 1998; Ortiz et al., 1999; Stoler et al., 1995; Zeng et al., 1999). However, none of these proteins have been implicated directly in the attachment of chromosomes to microtubules or in the generation of force.

Historically, an important question about chromosome-microtubule attachment has been the identity of the kinetochore-associated motors. In animal cells, the kinesin-related motor proteins (KRPs) CENP-E and MCAK have been shown to function in kinetochore-dependent chromosome movement, as has dynein (for review, see Rieder and Salmon, 1998); in yeast, it is not known which among the six KRP and dynein motors are kinetochore bound. Moreover, experiments in several organisms have shown that both the ATP-dependent sliding of motor proteins along microtubules and the GTP-dependent depolymerization of microtubule fibers are capable of generating sufficient force to move chromosomes (Hunter and Wordeman, 2000). Thus, nonmotor microtubule associated proteins (MAPs) may function to link kinetochores and microtubule plus ends during periods of polymer growth and shrinkage. The goal of a molecular analysis of yeast kinetochores is therefore to provide answers to the following general questions (1) how many different proteins are involved in chromosome-microtubule attachment and what are the relative roles of motors and MAPs, (2) do these proteins function only at kinetochores or also in other cellular structures, and (3) do different proteins mediate different aspects of the complex pattern of metaphase and anaphase chromosome movement?

The identification of microtubule binding proteins in yeast kinetochores has been hindered by the absence of an assay to monitor chromosome-microtubule attachment. However, we and others have recently shown that force-generating processes at *S. cerevisiae* kinetochores impose sufficient tension on paired chromatids during metaphase to transiently separate centromeric chromatin toward opposite ends of the spindle (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000). Transient separations can pull sisters up to 1 μm apart for several minutes (a large movement relative to the 1.5 to 2 μm yeast spindle) and involve toward-the-pole separating forces and opposing cohesive forces. We reasoned that, by exploiting the phenomenon of transient sister separation to measure and analyze forces exerted on centromeres in wild-type and mutant yeast strains, a molecular analysis of microtubule attachment would be possible. In this paper, we identify as kinetochore components ten *S. cerevisiae* proteins previously thought to be involved in other mitotic processes. Some of these proteins, or their mammalian orthologs, are motors or microtubule binding proteins and mutations in several of these newly-identified kinetochore subunits impair force generation and chromosome movement in vivo. These data lead us to conclude that we have identified some of the proteins involved directly in the formation of microtubule attachment sites.

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Results

We have previously reported that kinetochore proteins in *S. cerevisiae* localize during metaphase to two lobes that lie on either side of the spindle midzone (He et al., 2000). The separation between these lobes is typically about half the separation between spindle pole bodies (SPBs). The distribution of kinetochore proteins changes subtly on a time scale of seconds concomitant with fluctuations in the extent of overlap among the 32 centromeres in a mitotic haploid cell. Because the bilobed distribution of kinetochores was unexpected, we reasoned that it should be possible to identify additional kinetochore proteins based on this distinctive pattern and that some of these proteins might have been misidentified previously as spindle components. Of twenty or so spindle proteins whose functions were not well understood but for which some localization data were available, we found a total of ten (Table 1) that probably constitute structural kinetochore components and one kinase that appears to regulate kinetochore function.

To determine the localization of candidate kinetochore proteins, they were tagged with GFP at their extreme C termini and introduced into cells in the place of the wild-type gene using homologous recombination. The positions of the SPBs were determined by replacing the integral SPB protein Spc42p with a fusion to cyan fluorescent protein (Spc42p-CFP; Donaldson and Kilmartin, 1996; He et al., 2000). Time lapse and fixed-cell fluorescence microscopy were performed in two colors to visualize both GFP fusion proteins and spindle poles using optical sectioning microscopy followed by iterative deconvolution (on an Applied Precision DeltaVision Microscope). Cell cycle state in asynchronous cultures and synchrony-release experiments were determined by examining nuclear morphology and spindle length. All GFP (or CFP) fusions tested, including those involving essential genes (Table 1), supported wild-type rates of growth. To demonstrate that various proteins were indeed localized to kinetochores, we examined the CBF3-dependence of their localization. GFP fusion proteins and Spc42p-CFP were introduced into *ndc10-1* cells and analyzed at 37°C, conditions that inactivate CBF3 and therefore disrupt kinetochore structure (Goh and Kilmartin, 1993).

As an independent assay for centromere association, we asked whether GFP-tagged proteins were bound to centromeric DNA in vivo as judged by formaldehyde crosslinking and chromatin immunoprecipitation (ChIP). Wild-type and *ndc10-1* cells carrying different GFP-tagged proteins were treated with formaldehyde, cells were lysed, and DNA sheared by sonication to an average of 200–500 bp. Immune complexes were isolated using anti-GFP antibodies and the fraction of coprecipitating DNA determined by PCR using primers specific for *CENIV* and a negative control *URA3* sequence.

Proteins Localized Primarily to Kinetochores

The first proteins we examined were Ndc80p, Spc24p, Spc25p, and Nuf2p. Ndc80p, Spc24p, and Spc25p were originally identified by MALDI-based microsequencing as proteins that cofractionate with yeast spindle pole bodies (SPBs) and localize, by immuno-EM, to the nuclear face of the SPB central plaque and to microtubules (Wigge et al., 1998). Nuf2p has been reported to be an SPB component based on its bilobed localization, but the small separation between the lobes seemed to us typical of a kinetochore protein (Osborne et al., 1994). When fixed and live cells were examined by 3D deconvolution microscopy, Ndc80p-GFP, Spc24p-GFP, Spc25p-GFP, and Nuf2p-GFP were seen during metaphase to localize to two lobes on either side of the spindle midzone to move toward the poles during anaphase (Figures 1A–1F and data not shown). In cells that lack functional CBF3 (*ndc10-1* cells at 37°C) the bilobed pattern was abolished and replaced by dim, uniform nuclear fluorescence (Figures 1C and 1F). The fluctuating bilobed distribution of Ndc80p-GFP, Spc24p-GFP, Spc25p-GFP, and Nuf2p-GFP was indistinguishable from that of Slk19p-GFP and Mtw1p-GFP, two bona fide kinetochore proteins that we and others have analyzed in some detail (Goshima and Yanagida, 2000; He et al., 2000; Zeng et al., 1999).

When assayed by ChIP, Ndc80p-GFP, Spc24p-GFP, Spc25p-GFP, and Nuf2p-GFP exhibited strong *NDC10*-dependent association to *CENIV* DNA but only background association with a negative control *URA3* fragment (Figures 1P and 1Q). ChIP has previously been used to localize Cse4p, Mif2p, Slk19p, and other proteins to kinetochores (Meluh and Koshland, 1997; Meluh et al., 1998; Zeng et al., 1999), but we were concerned about possible nonspecific binding of spindle proteins to CEN DNA. As negative controls, we therefore performed ChIP with three nonkinetochore spindle proteins fused to GFP: Tub1p, which encodes α-tubulin, Tub4p, which encodes the SPB-localized γ-tubulin, and Spc42p, a component of the SPB central plaque. None of these proteins associated to any significant extent with centromeric DNA, confirming the specificity of the ChIP reaction (Figure 1R).

These findings confirm very recent reports that Ndc80p, Spc24p, Spc25p, and Nuf2p are components of an evolutionarily conserved multiprotein complex that associates with kinetochores in several eukaryotic organisms (Janke et al., 2001; Wigge and Kilmartin, 2001). Careful examination of images of Ndc80p-GFP, Spc24p-GFP, Spc25p-GFP, and Nuf2p-GFP at different stages of the cell cycle with and without Spc42p-CFP to control for fluorescence bleedthrough showed only the bilobed localization typical of kinetochores and no association with spindle poles, spindle microtubules, or other nuclear structures (Figures 1A and 1F; data not shown). We have found that Dam1p, a microtubule binding protein previously shown to be involved in spindle (Hofmann et al., 1998; Jones et al., 1999) and kinetochore function (Cheeseman et al., 2001) is a fifth protein that associates with *CEN*-DNA in a *NDC10*-dependent fashion and that exhibits a localization in metaphase essentially identical to that of Ndc80p (data not shown). Overall, we conclude that Ndc80p, Nuf2p, Spc24p, Spc25p, and Dam1p associate with centromeres during mitosis in a CBF3-dependent manner and that kinetochores may be the only cellular structures with a significant level of these proteins as judged by GFP-tagging.

Proteins Bound to Kinetochores and to the Mitotic Spindle

Next, we examined three proteins that appeared, from careful examination of published images, to be at least

Table 1. Summary of the Kinetochore Proteins Analyzed in This Study

Protein[a]	Localization[b]					ChIP		Alleles Used in This Study	Homologs	Interactions[a,c,d]	Reported Functions[d]
	Kinetochores	Nuclear-MTs	SPB	Cortical-Tip	Cyto-MTs	In WT	In *ndc10-1*				
Ndc80p (E)	+	−	−	−	−	+	−	*ndc80-1* (Wigge et al., 1998)	HEC1 (Zheng et al., 1999)	2H: *SPC24, SPC19*	Chromosome segregation
Nuf2p (E)	+	−	−	−	−	+	−	*nuf2-61; -457* (Osborne et al., 1994)	Nuf2R (Wigge and Kilmartin, 2001)	2H: *CIN8, NDC80, SPC19*	Chromosome segregation
Spc24p (E)	+	−	−	−	−	+	−	none	*S pombe* C336.08 (Wigge and Kilmartin, 2001)	2H: *SPC25*	Chromosome segregation
Spc25p (E)	+	−	−	−	−	+	−	none	N/A	2H: *SPC24*	Chromosome segregation
Dam1p (E)	+	−	−	−	−	+	−	*dam1-1; -9; -11* (Cheeseman et al., 2001; Jones et al., 1999)	N/A	2H: NDC80, SPC34 SL: *cin8*Δ	Microtubule binding; Spindle integrity
Spc19p (E)	+	+	−	−	−	+	−	none	N/A	2H: *SPC34, NDC80*	Unknown
Spc34p (E)	+	+	−	−	−	+	−	none	N/A	2H: *SPC19*	Unknown
Stu2p (E)	+	+	+	+	−	+	−	*stu2-276; -277; -278; -279* (this work)	XMAP215	N/A	Microtubule binding
Bik1p (NE)	+	+	−	+	+	+	−	*bik1*Δ*:His3* (Berlin et al., 1990) (this work)	CLIP-170	2H: STU2 SL: *cin8*Δ	Spindle elongation
Cin8p (NE)	+	+	−	−	−	+	−	*cin8*Δ*:His3* (Hoyt et al., 1992)	BimC kinesins	SL: *bik1*Δ SL: *dam1-1*	Spindle assembly and elongation; Chromosome segregation
Ipl1p (E)	+	+	−	−	−	−	−	*ipl1-2, -321* (Biggins et al., 1999; Francisco and Chan, 1994)	Aurora-like kinases	SL: *cin8*Δ	Histone phosphorylation; Regulation of kinetochore-microtubule binding

[a] Abbreviations: E, Essential; NE, Nonessential; 2H, Two-Hybrid; SL, Synthetic Lethality; SP, Suppression.

[b] Cellular localization shown by this study.

[c] Only interactions among proteins listed in this table are shown. Extensive genetic and biochemical interactions among Ndc80p, Nuf2p, Spc24p, and Spc25p have also been demonstrated (Janke et al., 2001; Wigge and Kilmartin, 2001) and are not shown.

[d] References for interactions and reported functions are listed at the YPD database (Costanzo et al., 2000) and for 2-hybrid analysis in Ito et al., 2001 and Newman et al., 2000.

partially localized to two nuclear lobes: Spc19p and Spc34p, proteins that copurify biochemically with SPBs (Wigge et al., 1998) and Cin8p, one of the six kinesin-like proteins in budding yeast (Hoyt et al., 1992; Roof et al., 1992). GFP fusions of all three proteins showed some *NDC10*-dependent kinetochore localization (Figure 1) and specific binding to centromeric DNA by ChIP (Figures 1P and 1Q) However, in contrast to Ndc80p, Nuf2p, Spc24p, Spc25p, and Dam1p discussed above, Spc19p, Spc34p, and Cin8p also localized to other microtubule-based structures in the cell.

In wild-type cells, Spc19p, Spc34p, and Cin8p were broadly similar in being localized to two kinetochore-like lobes as well as along the microtubules of the mitotic spindle (Figures 1G–1O). In anaphase, all three proteins retained their spindle localization while also concentrating at spindle poles, where centromeres are clustered. As described previously, Cin8p also has the interesting property of localizing to the spindle midbody late in anaphase (Figure 1N; Hoyt et al., 1992). In *ndc10-1* cells, the bilobed components of Spc19p and Spc34p localization were abolished, whereas localization to spindle microtubules remained. Cin8p-GFP largely shifted to one pole or the other. The *NDC10*-dependence of localization is seen most clearly in intensity-distance plots that integrate the GFP and CFP signals along the spindle axis (see Figures 1J and 1L in particular). In interpreting the plots and images, it should be noted that spindles in *ndc10-1* cells are about 25% longer than in wild-type cells, reflecting the loss of kinetochore-dependent pulling forces that shorten the spindle. In conclusion, although the localization patterns of Spc19p, Spc34p, and Cin8p are more complex than those of the five proteins discussed in the previous section, imaging and

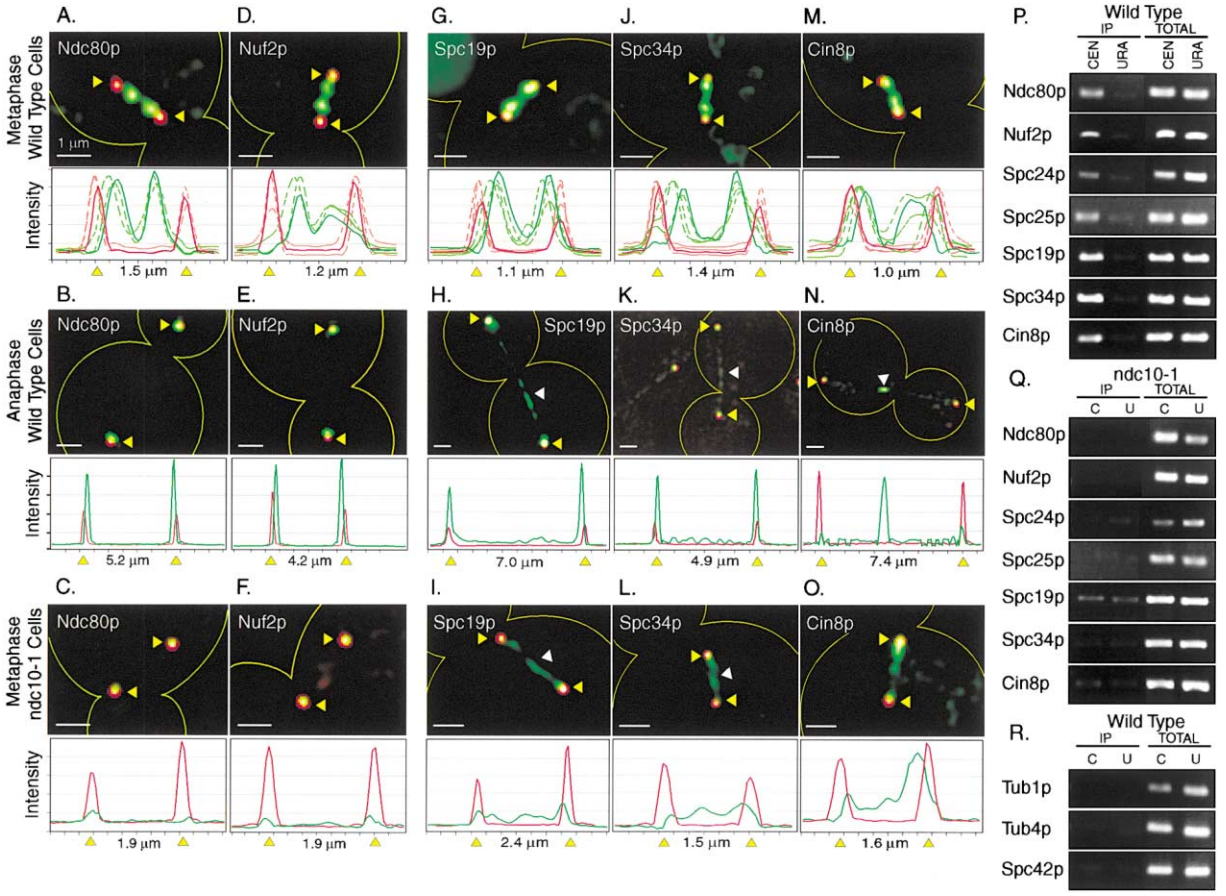

Figure 1. Analysis of Proteins Localized to Kinetochores, or to Kinetochores and Spindle Microtubules

Yellow arrows mark SPBs and white arrows spindle microtubules. (A, D, G, J, and M) Typical images from metaphase wild type cells carrying Spc42p-CFP (in red) and Ndc80p-GFP, Nuf2p-GFP, Spc19p-GFP, Spc34p-GFP, or Cin8p-GFP (in green). Images represent projections of 3D image stacks containing ten to twenty 0.2 μm sections. The graph shows the distributions of CFP and GFP signal intensities along the spindle axis (in arbitrary units) in several cells. The bold line is derived from the image shown after correction for bleed-through from the CFP to the GFP channel (see Experimental Procedures). (B, E, H, K, and N) Typical images from anaphase wild-type cells or (C, F, I, L, and O) metaphase *ndc10-1* cells. (P and Q) Crosslinking of proteins tagged with GFP to *CENIV* DNA in wild-type or *ndc10-1* cells at 37°C as assayed by chromatin immunoprecipitation (ChIP). DNA in immune complexes (IP) was amplified with primers specific for *CENIV* (CEN lanes) or, as a negative control, *URA3* (URA lanes) and compared to the amount of DNA in whole-cell lysates (TOTAL). (R) ChIP assays with control proteins not found at kinetochores. TUB1 encodes α-tubulin, TUB4, γ-tubulin, and SPC42, an integral component of the SPB.

ChIP are consistent with the idea that a fraction of Spc19p, Spc34p, and Cin8p is associated with kinetochores in metaphase yeast cells. Kinetochore association appears to be CBF3-dependent, whereas binding to spindle microtubules is CBF3-independent.

Proteins Bound to Kinetochores and a Variety of Microtubule-Based Structures

Next, we examined two microtubule binding proteins that are found in both the cytoplasm and the nucleus: Bik1p and Stu2p. Bik1p is homologous to the plus-end microtubule binding protein mammalian CLIP170 (Berlin et al., 1990) and Stu2p is a microtubule binding protein similar in sequence to *Xenopus* XMAP215 (Wang and Huffaker, 1997) and human TOGp (Spittle et al., 2000). Bik1p-GFP exhibited a complex localization to kinetochore-like lobes, to spindle microtubules, and to distinct spots in the cytoplasm that correspond to cortical attachment sites (Berlin et al., 1990), and only part of the localization appeared to be disrupted in *ndc10-1* cells at 37°C (Figures 2A and 2C). Cortical capture sites are structures in the plasma membrane that bind the plus ends of microtubules that emanate from SPBs and function to orient the nucleus in the mother-bud neck late in metaphase (for review see Bloom, 2000). The localization of Bik1p to both kinetochores and cortical attachment sites is consistent with data that CLIP-170 binds selectively to the plus ends of microtubules. Stu2p-GFP was found in a pattern broadly similar to that of Bik1p and again, only a subset of the nuclear Stu2p-GFP appeared sensitive to *ndc10-1* inactivation (Figures 2D–2F). To determine whether the bright spots of Stu2p-GFP along the periphery of the cell might be cortical capture sites, we generated cells carrying α-tubulin-GFP (Tub1p-GFP) and Stu2p-CFP. In both metaphase and anaphase cells, cytoplasmic foci of Stu2p-CFP clearly lay at the extreme ends of cytoplasmic microtubule bundles, strongly suggesting that the foci were

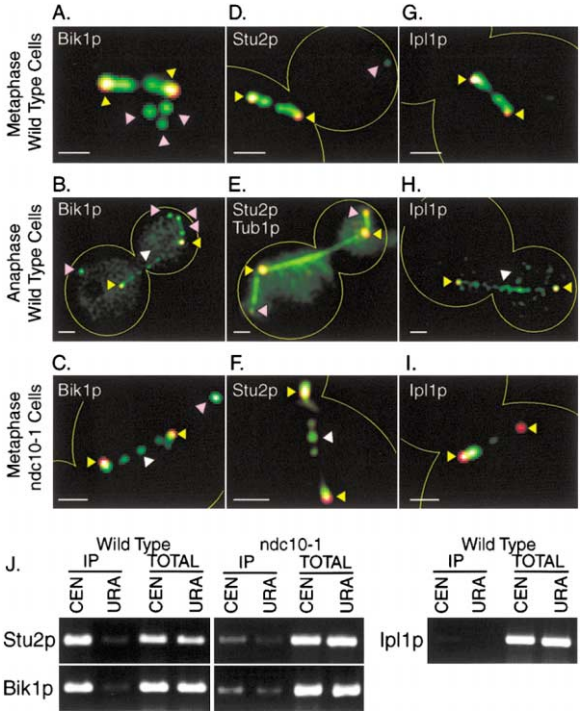

Figure 2. Analysis of Proteins Localized to Kinetochores and to Other Microtubule-Based Structures

(A–I) Imaging of Bik1p, Stu2p, and Ipl1p as described in Figure 1. Yellow arrows mark SPBs, white arrows mark spindle microtubules, and pink arrows mark cortical capture sites. Note that in (E), the fluorophores have been changed so that Stu2p is fused to CFP (in red) and α-tubulin to Tub1p to GFP (in green). (J) Crosslinking of Bik1p, Stu2p, and Ipl1p to *CENIV* DNA as assayed by ChIP as described in Figure 1.

indeed cortical capture sites. Consistent with this localization, Stu2p and the well-characterized cortical site protein Kar9p have recently been shown to interact by two-hybrid analysis (Miller et al., 2000). By ChIP, both Bik1p and Stu2p exhibited *NDC10*-dependent binding to centromeric DNA (Figure 2J). From these data, we conclude that Bik1p and Stu2p are proteins that associate with a variety of microtubule-based structures including kinetochores and cortical capture sites, both of which bind to microtubule plus ends.

The final protein we examined was Ipl1p, an Aurora kinase that has been proposed to function in yeast kinetochore assembly (Biggins et al., 1999; Sassoon et al., 1999). It has previously been observed that Nuf2p is mislocalized in *ipl1-2* cells, a finding interpreted to reflect a role for Ipl1p in SPB formation (Kim et al., 1999). However, since Nuf2p is actually localized to kinetochores and not SPBs as previously assumed, we wondered whether Ipl1p might be a regulator of kinetochores. With Ipl1p-GFP, we observed a pattern consistent with kinetochore localization as well as with spindle binding, and a subset of the localization was *NDC10*-dependent but CEN-association was not detected by ChIP. The ChIP assay is a stringent criterion for kinetochore association and some kinetochore proteins may simply be too distant from DNA to be successfully crosslinked by formaldehyde. Our inclusion of Ipl1p in this analysis is justified by its clear role in chromosome movement (see below).

Mapping Chromosome Association to Centromeric DNA

We have previously proposed that centromeric chromatin in budding yeast spans nearly 20 kb of DNA centered on the centromere. This centromeric chromatin appears to be involved in the large-scale stretching that occurs during transient sister separation. It seemed possible that proteins we had localized to centromeres might be associated not with kinetochores themselves, but rather with an extended chromatin domain. This seemed particularly likely for Ndc80p, whose human homolog, Hec1, has been shown to complement an *NDC80* disruption in single copy and to interact biochemically and genetically with the Smc1p and Smc2p subunits of yeast cohesin and condensin (Zheng et al., 1999, 2000). In mitotic *S. cerevisiae* cells, cohesin is found both at centromeres and at discrete sites along chromosome arms, whereas condensin is ubiquitously distributed along chromatin (Freeman et al., 2000; Megee et al., 1999; Tanaka et al., 1999). To map the sequences to which Ndc80p binds, we used ChIP to quantitate its association to five successive 200 bp fragments of chromosomal DNA that span *CENIII*. We also examined Ndc80p binding to an 18 kb region on the arm of chromosome V that has previously been shown to contain a cohesin binding site (Tanaka et al., 1999). We observed that Ndc80p was present at high levels on the 200 bp fragment centered on *CENIII*, at much lower levels on sequences to the left and right of the centromere, and at only background levels at sites along chromosome V arms (Figures 3A and 3B). The concentration of Ndc80p at centromeres was, if anything, tighter than that of Mif2p (Meluh and Koshland, 1997), the centromere-bound homolog of mammalian CENP-C, and clearly distinct from the broad distribution of Scc1p, a cohesin subunit (Megee et al., 1999; Tanaka et al., 1999). We therefore conclude that Ndc80p is tightly concentrated at centromeres and does not have the broader distribution along chromosomes characteristic of cohesin and condensin. Similar CEN-specific crosslinking was observed for Spc19p, Spc34p, Cin8p, Bik1p, and Stu2p (Figure 3C), confirming our conclusion that all of these proteins are specifically associated with kinetochores.

Mutations in Kinetochore Proteins Reduce Transient Sisters Separation

To establish a function for proteins localized to kinetochores, we examined the extent of transient sister chromatid separation. We reasoned that mutations in proteins required for chromosome-microtubule attachment should interfere with the imposition of tension on sister centromeres and thereby decrease the frequency or extent to which sisters separate in metaphase. Nine of the eleven proteins we had localized to kinetochores are essential for vegetative growth but temperature sensitive mutants were available for only four (Table 1). Among the five genes without conditional mutations, it seemed most important to examine Stu2p, because it has a higher cell homolog (XMAP215) whose function is at least partially understood (Tournebize et al., 2000). We therefore generated 19 temperature sensitive alleles in the *STU2* gene using PCR mutagenesis and plasmid shuffling (see Experimental Procedures), and selected

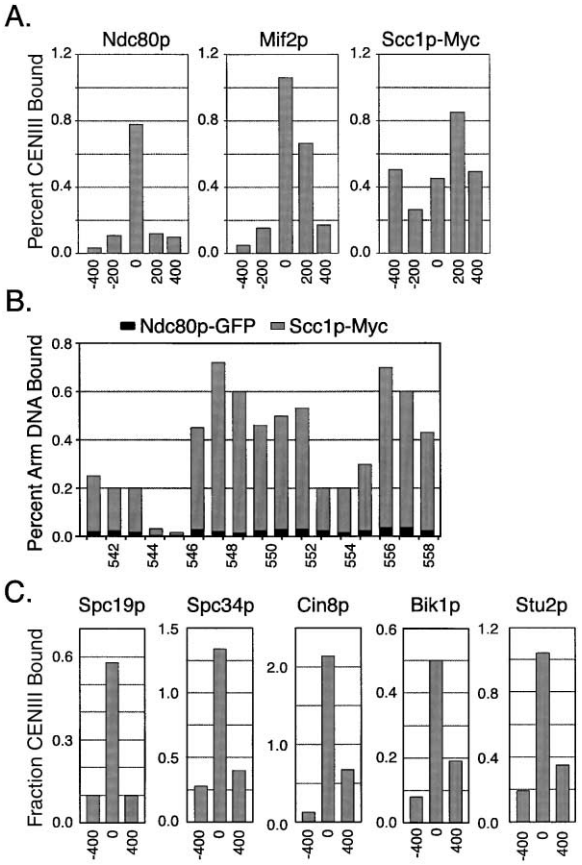

Figure 3. Quantitative Crosslinking Analysis by ChIP

(A) Quantitative ChIP analysis of the binding of Ndc80p, Mif2p, and Scc1p to *CENIII* and flanking DNA. The horizontal axis denotes the position of 200 bp fragments, relative to the center of *CENIII*, and the vertical axis the fraction of total DNA present in immune complexes as determined by PCR from serial dilution. Ndc80p was immunoprecipitated with anti-GFP polyclonal antiserum, Mif2p with rabbit polyclonal antiserum, and Scc1-Myc_{12} with anti-Myc polyclonal antibodies.

(B) Quantitative ChIP analysis of Ndc80p and Scc1p crosslinking to sites along the arm of chromosome V (Tanaka et al., 1999). Numbers on the horizontal axis refer to the position, in kilobases, from the left telomere as indicated at the Stanford Genome Database.

(C) Quantitative ChIP analysis of Spc19p, Spc34p, Cin8p, Bik1p, and Stu2p binding to *CENIII* and flanking DNA as described in (A).

for further analysis alleles that arrested within one cell cycle of temperature upshift.

Centromeric DNA was visualized by integrating a TetO array 2 kb from CEN IV (construct −2ChIV of He et al., 2000) in cells expressing TetR-GFP and a Spc42p-GFP. This generates cells in which both *CENIV*-proximal chromatin and spindle poles are marked with small green dots, permitting rapid single-color imaging. To quantitate sister separation in wild-type and mutant strains, they were synchronized at START using α-factor and then released into prewarmed medium at 37°C for 75–90 min prior to fixation and imaging. When the extent of synchrony was assessed morphologically, more than 90% of cells were observed to have entered prometaphase and assembled bipolar spindles, and fewer than 5% were in anaphase. As positive and negative controls, we showed that the fraction of wild-type cells with separated sisters was 50%–60%, whereas in *ndc10-1* cells it was less than 1% (Figure 4A; He et al., 2000). In *ndc80-1*, *dam1-1*, *nuf2-61*, and *stu2-277* cells, the extent of transient sister separation was 10- to 20-fold lower than in wild-type cells, while in *ipl1-321* cells it was 4- to 5-fold lower (in about 15% of *ipl1-321* cells, centromeres appeared hyperstretched, with the TetO/TetR-GFP tag extended the full length of the spindle). Both *bik1*Δ and *cin8*Δ cells (which were assayed at 25°C, a temperature at which wild-type cells had fewer transient separations) exhibited a normal frequency of transient separation (Figure 4B). In conclusion, these data show that Ndc80p, Nuf2p, Dam1p, Stu2p, and Ipl1p are required for transient sister chromatid separation, and thus, probably, for the imposition of normal tension on sister chromatids.

The proteins in this study fall into two classes: those that are found primarily at kinetochores (as judged by imaging GFP fusion proteins) and those that localize to both kinetochores and other microtubule-based structures in mitotic cells. To determine whether this distinction is also reflected in the functions of the proteins, we asked whether mutations that reduce tension across sister kinetochores also impair the migration of the nucleus into the mother-bud junction, an essential step in mitosis mediated by the interaction of cytoplasmic microtubules with cortical attachment sites. We observed that whereas nuclear migration was substantially perturbed in *stu2-276* cells, it appeared normal in *ndc10-1*, *ndc80-1*, *dam1-1*, *ipl1-321*, and *nuf2-61* mutants (Figure 4C; normal nuclear migration is observed in *bik1*Δ cells, a consequence of functional redundancy in proteins required for cortical attachment; Bloom, 2000). We conclude that kinetochore function is not required for nuclear migration and that the requirement for Stu2p probably reflects its localization to cortical capture sites. Additional nonkinetochore functions for proteins analyzed in this paper are summarized in Table 1.

Live Cell Analysis Reveals Three Types of Defect in Chromosome Movement

In principle, different kinetochore defects, ranging from complete failures of assembly to more subtle problems with force generation could reduce transient sister separation. To investigate the defects in *ndc80*, *nuf2*, *stu2*, *dam1*, and *ipl1* mutants, cells carrying GFP-tagged chromatids and SPBs were filmed at 37°C and the motion of centromeres determined relative to the spindle axis by manual and automated analysis of deconvolved 3D image stacks (Figures 4D and 4E; He et al., 2000). In wild-type cells, metaphase chromosome movement is characterized by several superimposed motions along the spindle axis: rapid oscillations 10%–20% of spindle length of 0.2–0.5 μm, long duration oscillations 30%–60% of spindle length of up to 1.0 μm, and transient sister separations in which the two chromatids move independently for periods of 2–10 min (He et al., 2000). In neither *ndc10-1*, *ndc80-1* (Figure 4F and 4H, green and orange lines, respectively), or *nuf2-457* cells (Figure 4G, blue lines; see figure legend for additional details) were any of these behaviors observed during metaphase and chromosomes remained exclusively in the mother cell at anaphase. Furthermore, whereas chromosomes

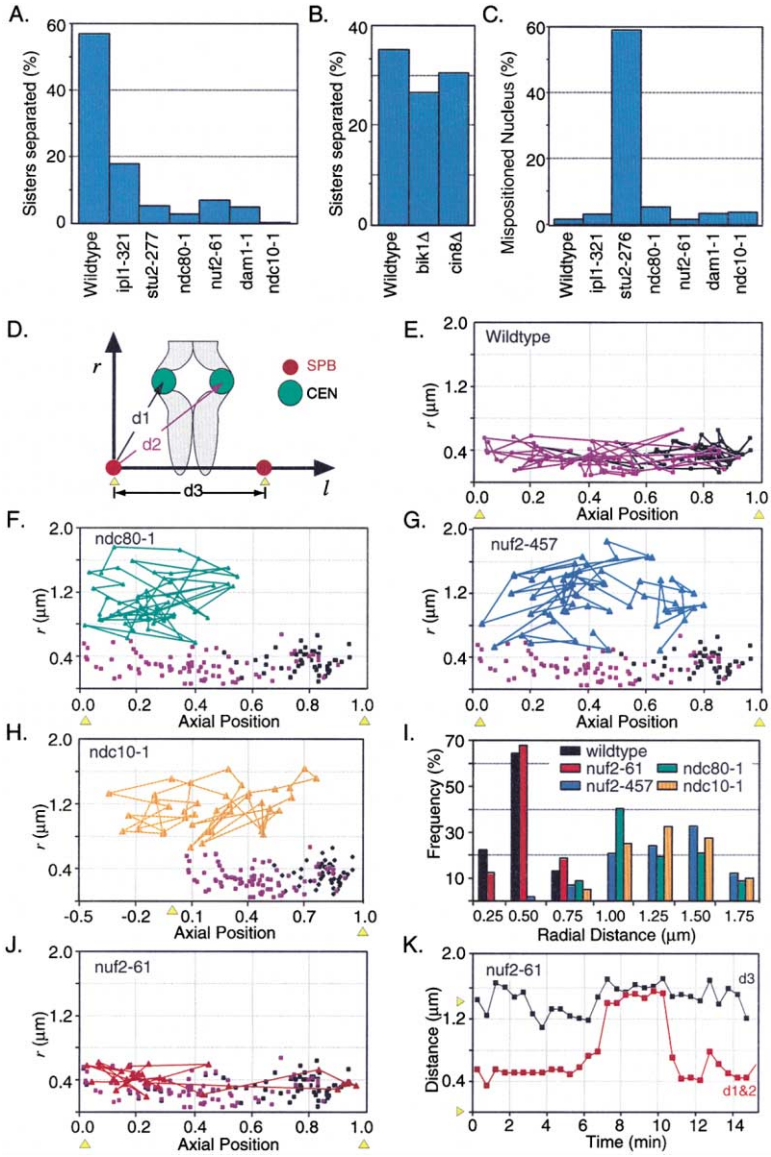

Figure 4. Effects of Mutations in Kinetochore Proteins on Transient Sister Separation, Nuclear Positioning Migration, and Chromosome Movement

(A) Mutations in essential kinetochore proteins reduce transient sister separation. Cells carrying Spc42p-GFP, the centromere-proximal −2ChIV chromosome tag (He et al., 2000), and the indicated temperature sensitive mutations were synchronized at START with α-factor at 25°C, and then released into prewarmed medium at the restrictive temperature of 37°C for 75 min prior to fixation and analysis. The fraction of metaphase cells (n ≈ 100), as judged by nuclear morphology, with separated sister chromosome tags was then scored.

(B) Analysis of transient sister separation in wild-type, *bik1Δ::HIS3*, and *cin8Δ::HIS3* cells performed at 25°C, as described in (A).

(C) Analysis of nuclear positioning prior to anaphase. Preanaphase cells in which the nucleus had not migrated to the bud-proximal hemisphere of the mother cell were scored as mispositioned (Korinek et al., 2000). Note that the *STU2* allele used in this analysis was different from that in (A). We have observed partial separation of *STU2* function in these alleles, with *stu2-277*; *279* having the greatest defect in transient sister separation and *stu2-276* having the greatest defect in nuclear positioning (by comparison 10% of *stu2-276* cells were transiently separated in a synchrony-release experiment).

(D) Schematic of the −2ChIV GFP chromosome tag and Spc42p-GFP spindle pole tag, showing the spindle-centered polar reference system (with *r* and *l* coordinates) and three key parameters: d1, the distance from the reference spindle pole to the center of one chromatid tag; d2, the distance to the sister chromatids tag; and d3, the SPB-SPB distance.

(E) Scatter plot of the positions of the −2ChIV chromosome tag over 15 min in a time-lapse movie of a wild-type cell. The vertical axis is the absolute radial position (*r*) and the horizontal axis the relative axial position (*l*/*d3*). Dots represent the position of the tag at each time point; black and purple denote the independent paths of the two sisters during periods of transient sister separation.

(F, G, and H) Scatter plots of chromosome movement in *ndc80-1*, *nuf2-457*, and *ndc10-1* cells compared to the wild-type movement in (E) (black and purple dots). Negative values of *l* arise when the CEN tag drifts so far from the SPBs that it is "behind" them. The phenotype of *ndc10-1* and *ndc80-1* cells are very homogeneous, but 15%–20% of *nuf2-457* cells exhibited behavior similar to that shown in (J) and (K). Synchrony-release experiments established that these cells are ones in which bipolar microtubule attachments had formed prior to temperature upshift.

(I) Distribution of radial distances (*r*) between the chromosome tag and the spindle axis in wild-type, *nuf2-61, -457*, *ndc10-1*, and *ndc80-1* cells. The data from sections (E–H) and (J) were used to calculate values for *r*, which were then grouped in bins of 0.25 μm to generate a frequency distribution. Scatter (J) and distance (K) plots for *nuf2-61* cells at 37°C. Because no transient separation occurs, d1 = d2 (red line). Spindle length (d3) is shown in black.

stayed within 0.40 μm of the spindle axis in wild-type cells, in *ndc10-1*, *ndc80-1*, and *nuf2-457* cells, they appeared to detach completely from the spindle and were typically 1.0–1.5 μm from the spindle axis, the maximum distance possible in 2.5 to 3.0 μm nucleus (Figure 4I). We conclude that loss-of-function mutants in *NDC80* and *NUF2*, like mutations in CBF3 genes, cause chromosomes to detach from spindle microtubules and move randomly within the mother cell.

In *ndc80-1*, *nuf2-457*, and *ndc10-1* cells, spindles undergo anaphase B elongation on approximately the same schedule as wild-type cells (data not shown), consistent with the conclusion by Janke et al. (2001) that the Ndc80p-Nuf2p complex is required for the mitotic checkpoint. We were therefore surprised to observe that at 37°C, *nuf2-61* cells arrested homogeneously at the metaphase-anaphase transition in a Mad1-dependent fashion (data not shown), implying that the *nuf2-61* lesion engages the mitotic checkpoint (see also Janke et al., 2001). When *nuf2-61* cells were examined by live-cell microscopy, chromosomes were observed to associate with one pole for 5 to 10 min and then suddenly jump

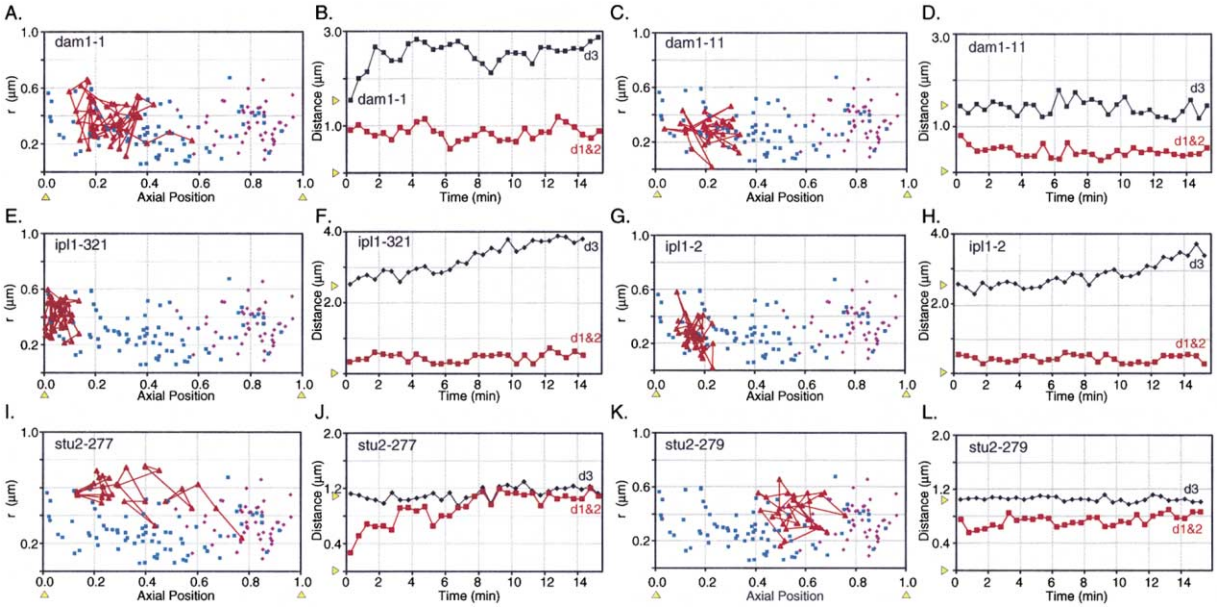

Figure 5. Distinct Defects in Chromosome Movement Are Observed in Cells Carrying Mutations in Different Kinetochore Proteins

(A, C, E, G, I, and K) Scatter plots showing the radial (*r*) and relative axial positions (*l*/*d3*) of a −2ChIV chromosome tag over a 15 min period of metaphase in various strains at 37°C (see Figure 4D for an explanation of the coordinates). The blue and purple dots show wild-type positions.

(B, D, F, H, J, and L) Distance plots transforming the data to show distance between the sister chromatids and a reference SPB (red line; because no transient separation occurs, d1 = d2) and the SPB-SPB distance (d3; black line). Multiple movies (3–6) were examined for each strain and the behaviors shown here are typical. The penetrance of the *ipl1-321* allele was only about 80% however, and some cells exhibited a sustained hyperstretching of the chromosome tag. The sampling rates used to collect this data are too low to permit the accurate measurement of rapid movements, but we have estimated the rates of long wavelength motions by smoothing the curves and then calculating a first derivative. On this basis, we find that centromeres in *stu2-277*; *279* cells move at least 3- to 4-fold slower than in wild type cells.

to the other pole, binding to it for 5 to 10 min before jumping again (Figures 4J and 4K, red lines). In some cells, we observed up to four shifts between the poles in a 30 min period. During these jumps, chromosomes remained as close to the spindle axis as in wild-type cells, supporting the notion that they were bound to microtubules, albeit aberrantly (Figure 4I). Our interpretation of these findings is that whereas microtubule attachment sites are unable to assemble in *nuf2-457* cells, the attachments that form in *nuf2-61* mutants are metastable. In the complete absence of kinetochore-microtubule attachment, no checkpoint signal is generated whereas metastable kinetochore-microtubule attachments do signal the checkpoint and arrest cells at metaphase (see Discussion).

Next, we analyzed chromosome segregation in *stu2*, *dam1*, and *ipl1* cells. Because we were interested in gene-specific differences in phenotype rather than allele-dependent variation, multiple temperature sensitive mutants were examined for each gene. To identify loss-of-function mutants, alleles were ranked in severity based on the extent of transient sister separation, as judged using fixed-cell assays (as described in Figure 4A). With many alleles, we observed a significant level of transient sister separation (approximately 20%–40% of chromatids were split), presumably representing a hypomorphic phenotype, but for each gene we were able to select two strong mutants.

In *dam1-1* and *dam 1-11* cells, we observed close association of tagged chromosomes with a single spindle pole (Figures 5A–5D). During this monopolar association, the chromosomes were found within 0.2 spindle diameters of the pole, but continued to oscillate rapidly (with an apparent velocity up to 1 μm/min). An even more dramatic monopolar association was observed in *ipl1-321* and *ipl1-2* cells, in which chromosomes remaining within approximately 0.1 spindle diameters of the pole (Figures 5E–5H). This monopolar association was clearly distinct from the spindle detachment observed in *ndc10-1*, *ndc80-1*, or *nuf2-457* cells, and in none of the several dozen movies of *dam1* and *ipl1* cells were chromosomes further from the spindle axis than in wild-type cells (0.4–0.5 μm). Yet a third phenotype was observed in *stu2-277* and *stu2-279* cells (Figures 5I–5L). Chromosomes in *stu2* mutants moved to the middle of the spindle (congression), where they oscillated back and forth along the spindle axis (see especially Figure 5I), apparently having achieved bipolar attachment and remaining at a wild-type radius from the spindle axis, but they covered a total distance 2- to 3-fold less than in wild-type cells and exhibited peak velocities at least 3- to 4-fold lower (0.3–0.4 μm/min). This pattern of movement is consistent with diminished force generation of bipolar attached chromatid pairs.

In summary, live-cell analysis of chromosome dynamics in strains carrying mutant kinetochore proteins reveals at least three classes of defects: a complete failure of chromosome-microtubule attachment (in *ndc80*, *nuf2*, and *ndc10* cells), attachment to a single pole with a failure to undergo congression (in *dam1* and *ipl1* cells), and bipolar microtubule attachment with reduced rates of movement and reduced tension across sister centro-

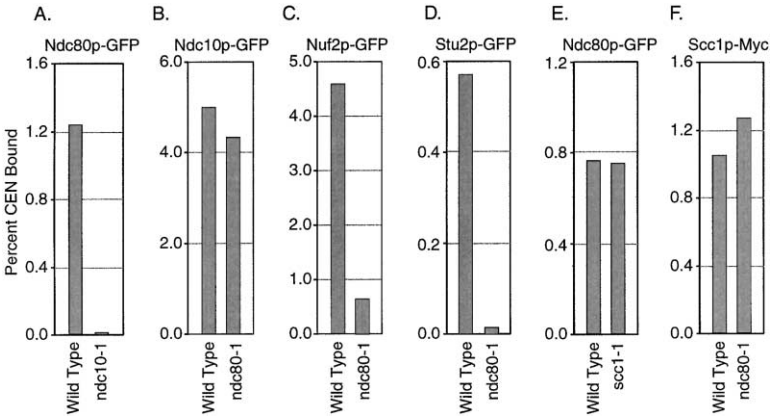

Figure 6. Hierarchy of Dependencies in the Association of Kinetochore Proteins with Centromeric DNA

Quantitative ChIP was used to determine the effects of various mutations on the extent to which GFP-tagged protein crosslinked to *CENIII* DNA. Cells were grown at 25°C to mid-log phase and shifted to 37°C for 2.5 hr prior to analysis. The amount of the *CENIII* DNA in immune complexes (IP) is shown as the percentage of the DNA in the lysate (see Figure 3). Absolute differences in the amount of DNA precipitated between panels are not considered to be meaningful.

meres (in *stu2* cells). The existence of distinct mutant phenotypes suggests that Ndc80p, Stu2p, Dam1p, Ipl1p, and Nuf2p have different functions in kinetochore-microtubule attachment.

A Multilayer Structure at the Yeast Kinetochore

Among the chromosome segregation defects described in this paper, those caused by *ndc10-1*, *ndc80-1*, and *nuf2-457* are much more severe than any others. The severe phenotype of *ndc10-1*, is thought to arise because no kinetochore components, not even error-detecting checkpoint proteins, can associate with centromeric DNA in the absence of CBF3 (Gardner et al., 2001; Goh and Kilmartin, 1993). To explore the role of Ndc80p in kinetochore assembly, we used ChIP to determine interdependencies among various kinetochore subunits. As described above, the association of Ndc80p with kinetochores was dependent on functional CBF3 (Figure 6A), but the reciprocal was not true: both the Ndc10p (Figure 6B) and Cep3p (data not shown) CBF3 proteins were centromere bound in *ndc80-1* cells at 37°C. However Nuf2p (Figure 6C) and Stu2p (Figure 6D) required both *NDC10* and *NDC80* function for kinetochore association. The association of the cohesin subunit Scc1p with centromeres requires *NDC10* (Tanaka et al., 1999), but Ndc80p and Scc1p bind independently to DNA (Figures 6E and 6F). Thus, the assembly of *S. cerevisiae* kinetochores appears to involve a hierarchy of dependencies: CBF3 at the first level, Ndc80p at the second, and Stu2p at the third. We conclude that the severity of the *ndc80* phenotype probably reflects a requirement for Ndc80p in the association of several other proteins with centromeres.

Discussion

We have used three criteria to identify kinetochore proteins: (1) CBF3-dependent localization to the yeast "metaphase plate," a bilobed distribution that fluctuates over time as kinetochores move back and forth along the spindle axis, (2) CBF3-dependent association with centromeric DNA, as judged by chromatin immunoprecipitation, and (3) functional involvement in kinetochore-microtubule attachment, as judged by disruption of the normal pattern of transient sister separation and chromosome movement. By these criteria, we find that ten previously described spindle proteins of uncertain function are associated with *S. cerevisiae* kinetochores and an eleventh appears to be a kinetochore regulator. Among these kinetochore proteins are several that bind to microtubules, or have animal cell homologs that are microtubule binding. Mutations in these proteins disrupt chromosome movement and appear to reduce or eliminate the tension that is normally imposed on sister centromeres. We hypothesize that the proteins are involved directly in the formation of microtubule attachment sites.

The mitotic spindle is a large multicomponent machine with long-range physical interactions among kinetochores, microtubules, and spindle poles. It is therefore important to inquire into the physical basis by which proteins might become associated with *CEN* DNA. We can imagine three possibilities. First, centromere association might be highly indirect, involving distant interactions mediated by microtubules. This seems unlikely because neither α-tubulin, γ-tubulin, nor the spindle pole component Spc42p detectably coprecipitated with CEN DNA by ChIP, and the available evidence suggests that ChIP is a reliable and highly selective cross-linking method (Meluh and Broach, 1999). Moreover, in cases in which CEN binding by ChIP is backed up by colocalization and functional data, our confidence in kinetochore association seems justified. Second, centromere association might involve binding directly to DNA, as in the case of CBF3, or binding to CBF3 in a multilayer kinetochore structure (Ortiz et al., 1999). In this case, a protein should be found associated with kinetochores independent of whether the kinetochores are linked to microtubules. Third, a protein that binds to microtubules might show centromere association through the attachment of microtubule plus ends to kinetochores. The human APC tumor suppresser protein, for example, localizes to kinetochores as a consequence of its binding to microtubule ends (Kaplan et al., 2001). Both of these latter possibilities are consistent with a protein's functioning in aspects of chromosome-microtubule attachment, but we have not yet distinguished between them experimentally.

Kinetochore-Associated Microtubule Binding Proteins

Among the eleven proteins discussed in this paper, four—Stu2p, Bik1p, Dam1p, and Cin8p—have been shown previously to bind microtubules and mutations in two of these—Stu2p and Dam1p—abolish the tension

normally imposed on sister kinetochores. In principle, mutations that affect transient sister separation could act by diminishing the microtubule-mediated forces that pull centromeres apart or by increasing the cohesive forces that hold sister kinetochores together. Although our data do not conclusively distinguish between these possibilities, the abnormal chromosome movements we have observed seem most consistent with a failure to establish and maintain microtubule attachment, and not with an increase in sister cohesion. We speculate that the failure of deletions in the nonessential *CIN8* and *BIK1* genes to impair kinetochore-microtubule attachment is a consequence of functional redundancy. Previously reported redundancy in *CIN8* and *KIP1* during spindle assembly (Hoyt et al., 1992) and *BIK1* and *KAR9* during nuclear positioning (Miller and Rose, 1998) support this conclusion.

The higher cell homologs of Stu2p, XMAP215 in *Xenopus* and TOGp in humans, have been shown to bind to and modulate the dynamic behavior of microtubules (Gard and Kirschner, 1987; Tournebize et al., 2000; Vasquez et al., 1994). Kinetochore-associated Stu2p might therefore be expected to contribute to chromosome movement by altering the stability of microtubule plus ends. A similar function has been proposed for Dis1, an *S. pombe* homolog of Stu2p that is required for correct chromosome movement and transient sister separation in fission yeast (Nabeshima et al., 1998). The human homolog of Bik1p, CLIP-170, localizes to microtubule plus ends, including those at kinetochores (Dujardin et al., 1998), and has also been postulated to regulate microtubule dynamics (Diamantopoulos et al., 1999). The *S. pombe* CLIP-170 homolog, Tip1p, functions to prevent catastrophic depolymerization of microtubule plus ends, thereby promoting their capture at the cell cortex (Brunner and Nurse, 2000). We might therefore expect that Bik1p participates in plus-end microtubule capture at *S. cerevisiae* kinetochores. Yeast Dam1p has been shown to bind to microtubules, but its biochemical analysis has just begun (Hofmann et al., 1998), and, Dam1p has no obvious homologs in higher cells.

The observation that Cin8p localizes to yeast kinetochores is surprising. Cin8p is one of four kinesin-like proteins in yeast with a nuclear function, but it is most similar to the BimC class of motors thought to slide microtubules relative to each other (for review, see Hildebrandt and Hoyt, 2000). This is not an activity expected of a kinetochore protein although a plus-end-directed motor could generate a force that separates spindle poles, a well characterized activity of Cin8p (Gheber et al., 1999; Hoyt et al., 1992). Assuming that the association of Cin8p with kinetochores is not adventitious, then its function must be redundant with that of other proteins. We have looked for kinetochore association by the Kip1-3 motors using GFP-tagging and ChIP and have preliminary data that Kip3p, at least, may also localize to kinetochores. We must now undertake a careful analysis of chromosome dynamics and transient separation in cells lacking combinations of two, three, and four motor proteins.

General Implications for Kinetochore-Microtubule Attachment

Four implications of general significance for kinetochore biology can be drawn from the data in this paper. First, multiple proteins appear to be involved in the attachment of kinetochores to microtubules, including both motor proteins and MAPs, and these proteins play at least partially overlapping roles. Mutations in *NDC10*, *NUF2*, and *NDC80* cause a complete disruption of chromosome-microtubule attachment, apparently because they disrupt kinetochore assembly. In contrast, mutations in *STU2*, *IPL1*, and *DAM1* interfere with chromosome movement but kinetochores retain some microtubule attachment. It therefore seems likely that multiple microtubule binding proteins contribute simultaneously to the formation of a fully functional attachment site.

Second, the formation of kinetochores with at least some microtubule binding activity, however aberrant, is required for mitotic checkpoint function. The genetic interaction between structural components of the kinetochore and the mitotic checkpoint is complex. Some mutations in kinetochore proteins, such as *ndc10* (Goh and Kilmartin, 1993; Tavormina and Burke, 1998) and *spc24* and *ndc80* (Janke et al., 2001; Wigge and Kilmartin, 2001), are as effective as *mad2*Δ in disrupting checkpoint function. Other mutations however, appear to cause a checkpoint-dependent arrest, including *ctf13* (Doheny et al., 1993) and *cep3* (Strunnikov et al., 1995). This difference cannot be explained simply by postulating different biochemical functions for checkpoint-disrupting and checkpoint-engaging mutations: Ndc10p, Ctf13p, and Cep3p are all required for the DNA binding activity of CBF3. However, our data on *nuf2-61* and *nuf2-457* provide strong support for the hypothesis that it is the extent of kinetochore disruption that determines whether the checkpoint will function. When chromosome attachment is completely disrupted, as seen in *nuf2-457* cells, the checkpoint is abolished. However, when metastable attachments are generated, as in *nuf2-61* cells, the checkpoint is engaged. Similarly, the partially defective attachments generated in *dam1* and *stu2* cells arrest cells in a checkpoint-dependent fashion. These data fit well with the idea that kinetochores are the source of a checkpoint signal that acts to monitor the formation of fully functional microtubule attachment sites (Gardner et al., 2001).

Third, mutations in different proteins give rise to different defects in chromosome movement, including a complete loss of attachment (in *ndc10*, *ndc80*, and *nuf2*), slow movement of chromatids that have apparently achieved bipolar attachment (in *stu2*), and close association with a single pole, presumably reflecting monopolar microtubule attachment (*dam1* and *ipl1*). This latter phenotype could arise either from a failure to duplicate kinetochores following DNA replication, or from a failure to develop microtubule attachments strong enough to oppose the splitting forces exerted on sisters during metaphase. A likely interpretation of these three phenotypic classes is that different proteins mediate different aspects of microtubule attachment and chromosome movement.

Fourth, many proteins involved in kinetochore-microtubule attachment also localize to other microtubule-based structures and appear to have more than one function in the cell. For example, Stu2p and Bik1p appear to function at both kinetochores and at cortical capture sites (and probably also at SPBs; Wang and Huffaker, 1997). Membrane-associated cortical capture sites bind cytoplasmic microtubules that emanate from

the SPB and function to orient the nucleus into the mother-bud neck, a precondition for transporting chromosomes into the daughter during anaphase B (for review, see Bloom, 2000). The important similarity between cortical capture sites and kinetochores is that both bind to the plus ends of dynamic microtubules.

In conclusion, the data in this paper suggest that yeast kinetochores contain several functional layers comprising DNA binding proteins such as CBF3, linker proteins such as Ndc80p, and microtubule binding components such as Stu2p, Dam1p, Cin8p, and Bik1p. The DNA binding and linker proteins seem to be highly specific to kinetochores, whereas several of the microtubule binding proteins have other functions in the cell. Although additional kinetochore subunits undoubtedly remain to be identified in yeast, our data have implications for the fundamental question of whether it is motor or nonmotor proteins that play the primary role in microtubule attachment. Our findings clearly point to a critical role for nonmotor MAPs in chromosome-microtubule binding and force generation.

Experimental Procedures

Yeast Strains and Manipulations

All yeast strains used in this study were haploid and derived from W303 or S228C. Chromosomes and spindle poles were tagged with GFP as described (He et al., 2000). Proteins were tagged with GFP as follows: a 400–1000 bp C-terminal gene fragment was amplified with PCR and EGFP linked at the C terminus in the integrating vector pRS306. The endogenous gene was replaced with the tagged form in one-step gene replacement and correct integrants confirmed by PCR. Nineteen *stu2* ts mutants were generated by mutagenizing the *STU2* ORF in vitro using error-prone PCR, replacing *STU2* in the genomes with the library of mutagenized clones and then complementing the ts phenotype with wild-type *STU2* on a plasmid. Sequencing revealed the presence of multiple mutations in each ts allele. ChIP experiments were performed using standard methods (Meluh and Broach, 1999), and quantitation of PCR products by serial dilution. Anti-GFP polyclonal antibodies were from Clontech, anti-myc polyclonal antibodies from Santa Cruz Biotechnology, and anti-Mif2 polyclonal antibodies produced in house.

Microscopy and Image Analysis

Live cell imaging was performed using a Deltavision optical sectioning microscope on a Nikon TE200 base and Roper RTE camera essentially as described in He et al., 2000. A custom-built heated stage and a Bioptechs lens heater with feedback control were used to maintain the temperature at 37°C (details are available upon request). Dual-color fixed cell images of cells carrying both CFP and GFP fusion proteins were collected using a Photometrics CH350 camera and Chroma 86002 JP4 (CFP) and 41018 (GFP) filters. These filters cause some leak-through from the CFP channel into the GFP channel. The intensity plots in Figures 1 and 2 were corrected for this bleedthrough.

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